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# An extremely hydroxyproline-rich glycoprotein is expressed in inverting *Volvox* embryos

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A sulphated glycoprotein, denoted I-SG, has previously been shown to be synthesized in *Volvox* under developmental control [(1982) FEBS Lett. 143, 311–315]. It is demonstrated that I-SG synthesis is restricted to a period during embryonic inversion as short as 10 min (out of the 48 h life cycle). This glycoprotein was purified and chemically characterized. 62% of its amino acid residues are hydroxyproline. Clusters of hydroxyproline residues were found to occur in its polypeptide chain. Besides arabinose and galactose, 3-*O*-methylgalactose and 6-*O*-methylgalactose were identified as constituents of I-SG.

(Volvox) *Inversion* *Arabinogalactan* *Hydroxyproline-rich glycoprotein* *Methyl sugar*

## 1. INTRODUCTION

Members of the genus *Volvox* are simple, multicellular, eucaryotic organisms and each individual is composed of only two cell types: somatic and reproductive cells. Asexual and sexual development of *Volvox carteri* has been described in detail [2,3]. During embryogenesis, 12 cleavage divisions of a reproductive cell generate all the cells of the adult organism. However, the orientation of the embryonic cells is the reverse of that found in the adult alga: the flagellar ends of the somatic cells are directed toward the interior of the embryo and the reproductive cells protrude from the surface rather than being embedded within the spheroid. At this stage of development the fascinating process of inversion turns the embryo completely inside-out. This morphogenetic movement takes less than 30 min.

The mechanics of inversion has been studied over many years [4–8]. These studies indicated that the basic mechanisms involved are essentially the same as those worked out for the gastrulating amphibian embryo [9,10]. Defined cell shape changes in a spatially and temporally programmed manner cause the curling of cellular sheets.

Different *Volvox* mutants exist which stop the inversion process at defined intermediate stages [11,12], indicating that a number of gene products control consecutive steps of this morphogenetic movement.

Previously, we presented evidence for the participation of two glycoconjugates in the inversion process [1,13]. One of these substances, the sulphated glycoprotein I-SG (inversion-specific sulphated glycoprotein) which is only synthesized during the very short period of inversion, was chemically characterized and the results are reported here.

## 2. EXPERIMENTAL

### 2.1. *Culture conditions*

*V. carteri* f. *nagariensis*, female strain HK 10, was obtained from the culture collection of algae at the University of Texas at Austin (R.C. Starr). The organism was grown in *Volvox* medium [14] at

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28°C in an 8 h dark/16 h light (8000–10000 lux) cycle [15].

2.2. *Pulse-labelling experiments*

2.2.1. 'Standard' procedure

Pulse labelling with $^{35}SO_4^{2-}$ was performed as described in [16,17].

2.2.2. Pulse-labelling of single *Volvox* embryos at the developmental stage of inversion

Single isolated *Volvox* embryos at the appropriate stage of inversion (selected under a dissection microscope) were transferred into sulphate-free *Volvox* medium and incubated in 30 $\mu$l with 1.1 MBq (30 $\mu$Ci) $^{35}SO_4^{2-}$ for 10 min. After addition of 40 $\mu$l SDS sample buffer [18] the sample was heated for 10 min at 95°C. The labelled extracts were analyzed on a 6% SDS-polyacrylamide gel.

2.3. *Isolation of embryos and somatic cell sheets*

Embryos at the appropriate developmental stage were isolated according to [17]. Embryo-free somatic cell sheets were isolated under a dissection microscope, after mechanical disruption of *Volvox* colonies.

2.4. *Purification of I-SG*

A 300 l *Volvox* culture (containing about 10 spheroids per ml) shortly after embryonic inversion was concentrated to about 600 ml by sieving over a 90 $\mu$m mesh nylon cloth. The suspension was brought to 1 M NaCl and stirred for 12 h at 4°C. The salt extract was adjusted to 1% Triton X-100 and 20 mM Tris-HCl (pH 8.0) and applied to a QAE-Sephadex A-25 column (60 ml bed volume) equilibrated with 1 M NaCl, 20 mM Tris-HCl (pH 8.0) and 0.01% Triton X-100. After the column was washed with 8 vols of the equilibration buffer, I-SG was eluted with a linear gradient of 1–2.4 M NaCl. I-SG containing fractions (detected by SDS-polyacrylamide gel electrophoresis and staining with 'Stains-all' [19,20]) were applied to a hydroxyapatite (Biorad) column (5 ml bed volume) equilibrated with 0.1% SDS in 10 mM Na phosphate (pH 6.5). After the column was washed with 5 vols of the equilibration buffer, I-SG was eluted with 100 mM Na phosphate (pH 6.5) containing 0.1% SDS. Final purification was achieved by preparative SDS-polyacrylamide (5%) gel electrophoresis. This procedure yields 0.5–1.0 mg homogeneous I-SG.

2.5. *Analytical determinations*

I-SG was hydrolyzed in 6 N HCl for 48 h at 100°C and analyzed in an automatic Biotronic LC 5000 amino acid analyzer. Deglycosylation was performed with anhydrous HF (Merck) as described [17]. The $M_r$ of deglycosylated I-SG was estimated on a Sephacryl-S300 column (68 × 0.9 cm) equilibrated with 0.1% SDS in 80 mM Na phosphate (pH 6.5). A mixture containing the [$^3$H]dansylated sample and standards was boiled for 2 min in the same buffer. Proteins were detected photometrically at 280 nm. Peptides for sequencing were derived by tryptic digestion according to [21]. 0.1 mg deglycosylated I-SG in 0.1 M *N*-methylmorpholinoacetate (pH 7.5) containing 2 M urea and 0.2 mM $CaCl_2$ was digested twice with 10 $\mu$g/ml trypsin for 4 h at 37°C. Tryptic peptides were separated by reversed-phase HPLC on a LiChrosorb-RP-18 column (Merck) applying a gradient from 0 to 40% acetonitrile in 0.1% trifluoroacetic acid (in 90 min) at a flow rate of 1 ml/min.

Sequence analysis of peptides was performed on an automated gas-phase amino acid sequencer (Applied Biosystems), and the PTH derivatives were detected by reversed-phase HPLC as in [22].

Conditions for hydrolysis of carbohydrates to monosaccharides, $C_1$ reduction and derivatization to alditol acetates and combined capillary GLC/mass spectroscopy were as described in [23,24]. Methylated sugars were separated from an acid hydrolysate by chromatography on a Biogel-P2 column (63 × 0.6 cm) equilibrated with 50 mM pyridine acetate (pH 5.5). Demethylation was achieved as in [25].

## 3. RESULTS

3.1. *Spatially and temporally controlled synthesis of I-SG*

I-SG was previously shown to be exclusively synthesized in *Volvox* spheroids containing inverting embryos. To localize the site of I-SG synthesis within a *Volvox* spheroid, the following experiment was performed. After pulse labelling with $^{35}SO_4^{2-}$, the *Volvox* spheroids were dissociated and separated by differential sieving into somatic cell

sheets and free embryos (section 2). Lysates from both cell fractions were analyzed on SDS-polyacrylamide gels for the presence of I-SG. As shown in fig.1A, I-SG is exclusively associated with the inverting embryo fraction.

Although it is known that I-SG synthesis is restricted to the period of embryonic inversion (about 25 min for a single embryo) we tried to define the period of I-SG synthesis with the highest possible resolution. To eliminate the problems introduced by asynchronously developing populations, $^{35}SO_4^{2-}$ pulse-labelling experiments (10 min) were performed with single embryos at each of the following stages of inversion: (i) at the time of phialopore opening, (ii) at the time of initiation of somatic cell sheet curling, (iii) at the time of halfway inversion and (iv) immediately at the end of the inversion process. The labelling patterns obtained (fig.1B) indicate that I-SG synthesis takes place only during the late phase of inversion, at about the beginning of the so-called snap-through stage of inversion [7]. Therefore, synthesis of I-SG is limited to a period shorter than 10 min within the 48 h life cycle of *Volvox*.

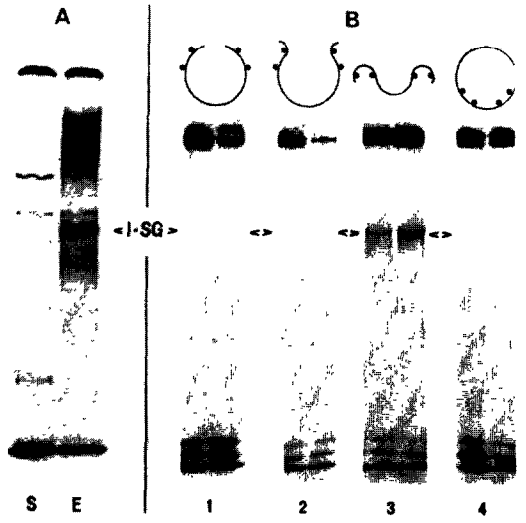


Fig.1. I-SG synthesis is spatially and temporally controlled. (A) *Volvox* spheroids containing inverting embryos were pulse labelled with $^{35}SO_4^{2-}$ (20 min) and then mechanically disrupted. Somatic cell sheets and free embryos were separated (section 2), lysed in SDS sample buffer and analysed on an SDS-polyacrylamide gel (5%). The fluorogram of the gel shows: S, somatic cell fraction; E, embryo fraction. (B) A single embryo at the desired stage of inversion was selected under a dissection microscope and pulse labelled with $^{35}SO_4^{2-}$ for 10 min (section 2) and lysed in SDS sample buffer. Pulse labellings were initiated at each of the stages schematically shown on top of the corresponding gel pattern. Each fluorogram shows the result of two independent experiments.

### 3.2. *Purification of I-SG*

I-SG was extracted from intact *Volvox* spheroids containing post-inversion embryos by incubation in 1 M NaCl for 12 h at 4°C. Even 0.3 M NaCl solutions are sufficient to elute a number of extracellular glycoproteins including I-SG from intact *Volvox* spheroids. Extracted I-SG was then adsorbed to QAE-Sephadex A25 and eluted with a linear NaCl gradient (1–2.4 M). The highly sulphated I-SG elutes between 1.4 and 1.8 M NaCl. Further purification was achieved by hydroxyapatite chromatography. At 100 mM sodium phosphate (pH 6.5), I-SG elutes from the hydroxyapatite column. At this stage of purification, I-SG is still contaminated with some material of lower $M_r$, which was removed by preparative SDS-polyacrylamide gel electrophoresis.

### 3.3. *Chemical characterization of the polypeptide chain*

Treatment of I-SG with anhydrous HF considerably reduces its apparent $M_r$ (fig.2), confirming the glycoprotein nature of I-SG. De-


Fig.2. Fluorogram of an SDS-polyacrylamide gel (12%) loaded with [$^3$H]dansyl-labelled I-SG before (1) and after (2) treatment with anhydrous HF for 90 min at 0°C.

glycosylated I-SG consists of a single polypeptide chain. Its amino acid composition is summarized in table 1. A remarkable feature of the amino acid composition is the extremely high content (62%) of hydroxyproline residues. Together with serine (11%) both these hydroxyamino acids constitute 73% of the amino acids within the I-SG polypeptide chain. The $M_r$ of the deglycosylated polypeptide chain was estimated by permeation chromatography on Sephacryl S300. For this purpose, the I-SG polypeptide chain was labelled by dansylation with [$^3$H]dansyl chloride and cochromatographed with phosphorylase *b* (92 kDa), carboanhydrase (30 kDa), myoglobin (17 kDa) and insulin (6 kDa) as internal molecular mass standards. I-SG polypeptide elutes from the column immediately after carboanhydrase, indicating a molecular mass of about 24 kDa.

Intact I-SG consists of 10–15 mol% amino acids and 85–90 mol% sugar residues. The deglycosylated (HF) polypeptide is degradable by trypsin, yielding peptides which can be separated by reversed-phase HPLC (fig.3). The most hydrophobic peptide (peak D in fig.3) turned out to be homogeneous and could be partly sequenced by automated Edman degradation. Peak C material could be sequenced after rechromatography on a TSK SP 5 column (LKB) using a gradient elution

Table 1

Amino acid composition of I-SG

| Amino acid | Residues/100 |
|---|---|
| Hyp | 62 |
| Asx | 3 |
| Thr | 2 |
| Ser | 11 |
| Glx | 4 |
| Pro | 0 |
| Gly | 2 |
| Ala | 3 |
| Val | 2 |
| Ile | 1 |
| Leu | 3 |
| Tyr | <1 |
| Phe | 1 |
| His | 1 |
| Lys | 2 |
| Arg | 3 |

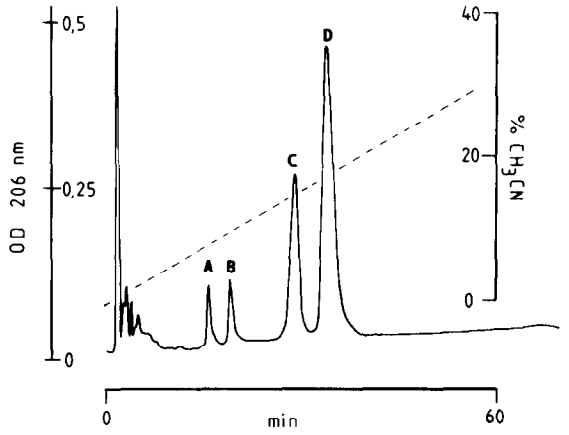


Fig.3. Isolation of tryptic peptides by reversed-phase HPLC derived from deglycosylated I-SG (section 2). Material in peak C and D was submitted to sequence analysis (table 2).

from 0 to 1 M NaCl in 10 mM K phosphate (pH 7; 1.0 ml/min in 55 min). The N-terminal amino acid sequences of tryptic peptides C and D are given in table 2. The most remarkable features of these sequences are repeating clusters of hydroxyproline residues.

### 3.4. *Carbohydrate composition of I-SG*

85–90 mol% of the I-SG molecule is carbohydrate. After acid hydrolysis, the neutral sugars of I-SG were analyzed as their alditol acetates by GLC-MS (fig.4A). Arabinose and galactose turned out to be the main sugar constituents, besides minor amounts of xylose, glucose, and mannose (table 3). In addition, two unusual sugar derivatives were present. From their mass spectra, these compounds were identified as a 6-*O*-methylhexitol and a 3- or 4-*O*-methylhexitol

Table 2

Amino acid sequences of the amino terminal end of tryptic peptides from I-SG

| | |
|---|---|
| Peptide C: | Val-Ala-Ser-Asn-Hyp-Ser-Hyp-Hyp-(Hyp)$_{>6}$- |
| Peptide D: | Leu-Arg-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Arg-Hyp-Hyp-Hyp-Hyp-Leu-Leu-Hyp-Gln-Ala-Hyp-Phe- |

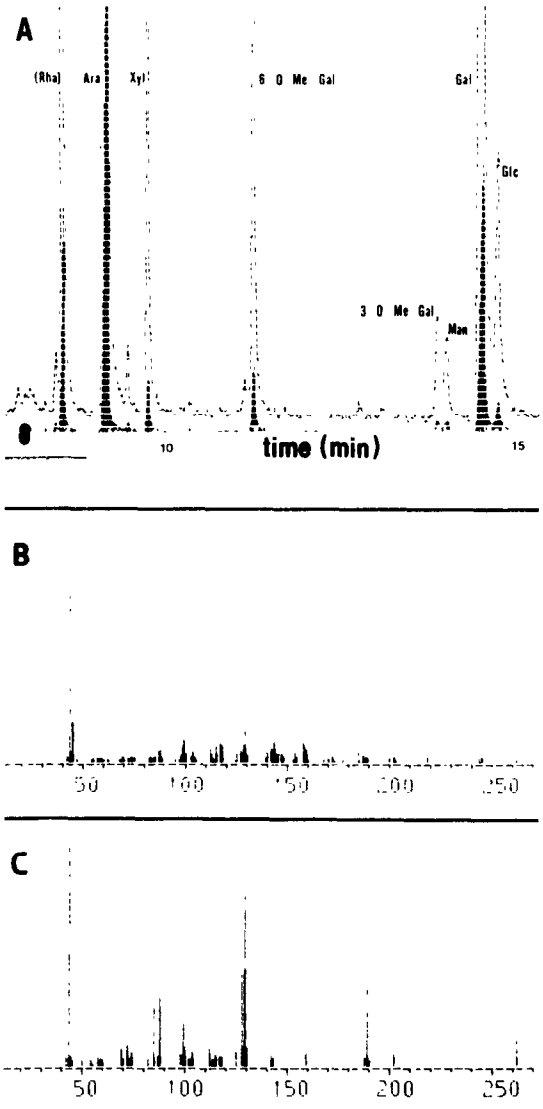


Fig.4. GLC-mass spectroscopy of neutral sugars derived from I-SG. I-SG was hydrolyzed to monosaccharides, reduced, peracetylated and submitted to GLC-mass spectroscopy. A, total ion profile (rhamnose was added as internal standard); B, mass spectrum of the peak marked 6-*O*-Me-Gal; C, mass spectrum of the peak marked 3-*O*-Me-Gal.

(fig.4B,C). These methylated sugars could be selectively separated from an acid hydrolysate of I-SG simply by chromatography on Biogel P2. The methylated sugars elute from the column well ahead of the monosaccharide fraction. After treatment of these methylated sugars with $BBr_3$, a procedure which removes the methyl residues, the resulting material was again analyzed by gas chromatography. After demethylation, only galactose is detectable in the chromatogram. Therefore I-SG contains 6-*O*-methylgalactose as well as 3- or 4-*O*-methylgalactose in considerable amounts (table 3). To discriminate between a 3- and 4-*O*-methylgalactose, the sugar hydrolysate was reduced with $NaBD_4$ instead of $NaBH_4$. From the resulting mass spectra the presence of 3-*O*-methylgalactose could be deduced. No amino sugars (amino acid analyzer) or uronic acids could be found to be present in I-SG.

Table 3

Sugar composition of I-SG

| Sugar | Residues/100 |
|---|---|
| Ara | 56 |
| Xyl | 4 |
| Man | 2 |
| Gal | 25 |
| 3-*O*-Methyl-Gal | 4 |
| 6-*O*-Methyl-Gal | 6 |
| Glc | 3 |

## 4. DISCUSSION

The present chemical data characterize I-SG as a hydroxyproline-rich glycoprotein of the arabinogalactan type. In the past few years growing evidence has been accumulated that the hydroxyproline-rich glycoproteins of the plant cell walls discovered by Lamport and Northcote [26] are of considerable importance in growth and development. From the work of Lamport's [27] and Varner's [28] group on extensin it is known that clusters of four hydroxyproline residues are repeated again and again over the whole polypeptide chain. In the case of I-SG even longer clusters of hydroxyproline residues seem to be a repeating structural motif. In fact, I-SG is the glycoprotein with the highest content of hydroxyproline (62%) known so far. Such a polypeptide should have a conformation very similar to the 'polyproline II' type which is a left-handed helix with a pitch of 0.94 nm with 3 residues per turn. Indeed, preliminary CD measurements as well as electron

microscopy (rotatory shadowing technique) support a rod-like conformation of I-SG. These data typify a structural rather than an enzymatic macromolecule.

Although methylated sugars are common constituents of algal polysaccharides (review [29]), the presence of methylated neutral sugars in a glycoprotein is an unusual observation, but has also been described to occur in isoagglutinins from *Chlamydomonas* [30]. Together with the high degree of sulphation, the saccharides of I-SG turn out to be highly modified.

I-SG synthesis is under strict developmental control. It is synthesized for less than 10 min within the 48 h life cycle of *Volvox*. This period coincides with the second half of the inversion process, a phase which was characterized as the snap-through stage of inversion. This close correlation supports the idea of a functional role of I-SG in inversion. Antibody preparations (polyclonal and monoclonal) against I-SG indeed inhibit the inversion process half-way, but unfortunately all preparations obtained so far cross-react with some other glycoproteins, reducing their experimental value.

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## REFERENCES

[1] Wenzl, S. and Sumper, M. (1982) FEBS Lett. 143, 311–315.
[2] Starr, R.C. (1969) Arch. Protistenk. 111, 204–222.
[3] Starr, R.C. (1970) Dev. Biol. Suppl. 4, 59–100.
[4] Kelland, J.L. (1964) PhD Thesis, Princeton University, Princeton.
[5] Pickett-Heaps, J.D. (1970) Planta 90, 174–190.
[6] Viamontes, G.I. and Kirk, D.L. (1977) J. Cell Biol. 75, 719–730.
[7] Viamontes, G.I., Fochtmann, L.J. and Kirk, D.L. (1979) Cell 17, 537–550.
[8] Green, K.J., Viamontes, G.I. and Kirk, D.L. (1981) J. Cell Biol. 91, 756–769.
[9] Holtfreter, J. (1943) J. Exp. Zool. 94, 261–318.
[10] Holtfreter, J. (1944) J. Exp. Zool. 95, 171–212.
[11] Huskey, R.J., Griffin, B.E., Cecil, P.O. and Callahan, A.M. (1979) Genetics 91, 229–244.
[12] Kirk, D.L., Viamontes, G.I., Green, K.J. and Bryant, J.L. jr (1982) in: Developmental Order: Its Origin and Regulation (Subtelny, S. ed.) pp.247–274, Liss, New York.
[13] Wenzl, S. and Sumper, M. (1986) Cell 46, 633–639.
[14] Provasoli, L. and Pintner, I.J. (1959) in: The Ecology of Algae (Tyron, C.A. and Hartmann, R.T. eds) Spec. Publ. no.2, pp.84–96, Pymatuning Laboratory of Field Biology, University of Pittsburgh.
[15] Starr, R.C. and Jaenicke, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1050–1054.
[16] Wenzl, S. and Sumper, M. (1981) Proc. Natl. Acad. Sci. USA 78, 3716–3720.
[17] Wenzl, S. and Sumper, M. (1986) Dev. Biol. 115, 119–128.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] King, L.E. and Morrison, M. (1976) Anal. Biochem. 71, 223–230.
[20] Green, M.R., Pastewka, J.V. and Peacock, A.C. (1973) Anal. Biochem. 56, 43–51.
[21] Allen, G. (1981) in: Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Burdon, R.H. eds) North-Holland, Amsterdam.
[22] Lottspeich, F. (1985) J. Chromatogr. 326, 321–327.
[23] Lechner, J., Wieland, F. and Sumper, M. (1985) J. Biol. Chem. 260, 860–866.
[24] Lechner, J., Wieland, F. and Sumper, M. (1985) J. Biol. Chem. 260, 8984–8989.
[25] Bonner, T.G., Bourne, E.J. and McNally, S. (1960) J. Chem. Soc., 2929–2934.
[26] Lamport, D.T.A. and Northcote, D.H. (1960) Nature 188, 665–666.
[27] Lamport, D.T.A. (1977) in: Recent Advances of Phytochemistry (Loewus, F.A. and Runeckles, V.C. eds) vol.II, pp.79–115, Plenum, New York.
[28] Chen, J. and Varner, J.E. (1985) EMBO J. 4, 2145–2151.
[29] Percival, E. and McDowell, R.H. (1981) in: Encyclopedia of Plant Physiology (Tanner, W. and Loewus, F. eds) Plant Carbohydrates II, vol.13B, pp.277–316, Springer, Berlin.
[30] Gerwig, G.J., Kamerling, J.P., Vliegenthart, J.F.G., Hofmann, W.L., Van Egmond, P. and Van den Ende, H. (1984) Carbohydr. Res. 127, 245–251.